Maternal metabolizable protein restriction during late gestation on uterine and umbilical blood flows and maternal and fetal amino acid concentrations near term in sheep

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Abstract

To examine the effects of maternal metabolizable protein (**MP**) restriction during late gestation on uterine and umbilical blood flows, conceptus size, and amino acid concentrations in the uterine and umbilical vessels, 11 ewes with singleton pregnancies were assigned to one of three isocaloric diets formulated to provide 60% of MP (**MP60**), 80% of MP (**MP80**), or 100% of MP (**MP100**) requirements from day 100 to 130 of gestation. On day 130 of gestation, intraoperative uterine and umbilical blood flows were obtained as well as serum samples from the uterine artery, uterine vein, umbilical artery, and umbilical vein. Ewes on the MP60 diet had lighter ($P = 0.04$) and smaller ($P \leq 0.05$) fetuses, but increased ($P = 0.02$) uterine blood flow relative to fetal weight compared with MP100 ewes, with MP80 being intermediate. Umbilical blood flow was similar ($P = 0.70$) across treatments. Glutamine, glycine, isoleucine, leucine, ornithine, serine, and valine concentrations were impacted ($P \leq 0.02$) by maternal treatment. While uterine flux of total serum nitrites was greater ($P = 0.03$) in MP60 and MP80 ewes compared with MP100 ewes, fetal flux did not differ. Decreased maternal protein intake resulted in less ($P < 0.01$) maternal cytochrome P450 1A enzyme activity. There were minimal impacts of maternal diet on steroid concentrations. Maternal dietary protein may alter fetal growth by impacting placental vasculature function and nutrient absorptive capabilities.

Key words: amino acids, metabolizable protein, sheep, umbilical blood flow, uterine blood flow

1. Introduction

Our laboratory has previously reported maternal nutrient restriction during mid to late gestation decreases umbilical and uterine blood flow near term in sheep (Lemley et al., 2012). Moreover, nutrient restriction decreases the amino acids (**AA**) concentrations in fetal circulation (Kwon et al., 2004; Satterfield et al., 2010; Lekatz et al., 2011; Lemley et al., 2013). The question remains if these alterations to blood flow and AA concentrations are due to an overall global restriction of nutrients or if there is a specific nutrient, or nutrients, driving these changes. Evidence from rodent models of maternal protein restriction demonstrates reduced offspring birth weight, elevated blood pressure in the offspring, and decreased vascular responses of the uterine artery (Langley et al., 1994; Langley-Evans and Jackson, 1996; Itoh et al., 2002). While much has been learned about the effects of maternal protein restriction in rodents, these findings cannot directly be applied to a ruminant, and data regarding the effects of protein restriction on uteroplacental function during gestation in ruminants is lacking.

Therefore, for the purpose of this study, isocaloric diets with varied amounts of metabolizable protein (**MP**) were evaluated. Metabolizable protein is defined by the NRC (2007) as the true protein, derived from dietary and microbial protein digested post-rationally and from which the constituent AA are absorbed from the intestine. Therefore, providing adequate MP during gestation may serve as a more appropriate indicator of how protein intake affects ruminant dams and their offspring.

We hypothesized isocaloric diets provided to dams with lower amounts of MP would decrease fetal growth by reducing uteroplacental blood flow, potentially mediated by sex steroids and/or nitric oxide, and nutrient delivery to the conceptus. Further, we

hypothesized MP restriction would reduce AA concentrations in uterine and umbilical vessels. Therefore, the objectives of this study were to evaluate the effects of isocaloric diets with varied levels of MP during late gestation (day 100 to 130) on conceptus development, uterine and umbilical blood flow, as well as sex steroid and AA concentrations in the uterine and umbilical vessels at day 130 of gestation.

2. Materials and Methods

Animal care and use was conducted according to protocols approved by the North Dakota State University (**NDSU**) Animal Care and Use Committee (#A0921).

2.1. Animals and experimental design

On ~ day 90 of gestation, 45 pregnant multiparous ewes were transported from the Hettinger Research Extension Center (Hettinger, ND, USA) to the Animal Nutrition and Physiology Center at NDSU (Fargo, ND, USA) where they were housed in individual pens (0.91 × 1.2 m) in an indoor facility until necropsy (130 ± 2 days of gestation). Within the facility, the temperature was held constant at 12°C, and lighting was controlled automatically (12:12-hour light-dark cycle with lights on at 0700 and off at 1900).

Ewes were acclimated to low-quality hay (Table 1) and the MP100 supplement (100% of the MP requirements, as determined by NRC, 2007; Table 2; Van Emon et al., 2014) for 10 days prior to starting dietary treatments. Ewes were weighed on two consecutive days (days 99 and 100 of gestation) prior to the initiation of treatments. On day 100 ± 2 of gestation ewes were randomly assigned to one of three isocaloric dietary

treatments (Table 2): **MP60**: 60% of MP requirements, **MP80**: 80% of MP requirements, and **MP100**: 100% of the MP requirements on a DM basis during the last 4 weeks of gestation (NRC, 2007; Van Emon et al., 2014). Dietary treatments were fed once daily at 0700 from 100 to 130 days of gestation. Ewes were given one hour to consume the supplement, which was always completely consumed, then low-quality forage (Table 1) was offered. There were no orts to collect throughout the study. Body weights were determined every 7 days throughout the dietary treatment period, and the amount of supplement and low-quality forage offered was adjusted for changes in body weight. All ewes had ad libitum access to fresh water and trace mineralized salt (4,000 ppm Zn, 1,600 ppm Fe, 1,200 ppm Mn, 325 ppm Cu, 100 ppm I, 40 ppm Co; American Stockman, Overland Park, KS).

2.2. Gestational day 130 intraoperative blood flow measurements

At 130 ± 2 days of gestation, a subset ($n = 12$; 67.0 ± 2.5 kg) of the 45 ewes carrying singletons (MP60: $n = 4$; MP80: $n = 4$; MP100: $n = 3$ [of this subset, one MP100 ewe was carrying twins and removed from the analysis]) underwent surgery to obtain intraoperative uterine blood flow via transonic blood flow probes and umbilical blood flow via Doppler ultrasonography as previously described by Lemley et al. (2012). Dams were weighed and anesthetized with 3 mg/kg of body weight sodium pentobarbital. Anesthesia was maintained via a jugular catheter. A catheter was placed into the maternal saphenous artery and advanced to the iliac artery via the femoral artery for monitoring and recording blood pressure throughout the procedure. The uterus was exposed via midventral laparotomy, covered with warm surgical towels, and a liberal

amount of physiological saline (37°C) was applied to the uterus every 5 min. The gravid uterine artery was located and a transonic flow probe (6 mm; Transonic Systems Inc., Ithaca, NY, USA) was placed around the gravid uterine artery prior to the first bifurcation of the uterine artery. Blood flow, as measured by the flow probe was recorded. Intraoperative measurements of umbilical artery hemodynamics were assessed using a duplex B-mode (brightness mode) and D-mode (Doppler spectrum) program of the color Doppler ultrasound instrument (model SSD-3500, Aloka UST-672). Umbilical artery hemodynamics were recorded by scanning the gravid uterine horn with a finger probe (5.0 MHz, Aloka UST-672; Aloka SSD-3500; Aloka America, Wallingford, CT) in B-mode until a clear image of the umbilical artery was located. Measurements were obtained by placing the cursor over the vessel in B-mode while simultaneously recording pulsatile waves in D-mode. The average angle of insonation for intraoperative umbilical artery blood flow was 62.5 ± 9.5 degrees (mean $\pm$ SEM). Three similar cardiac cycle waveforms were recorded and averaged per ewe. Cardiac cycle waveforms were plotted in D-mode by velocity (cm/s; y-axis) and time (s; x-axis). Umbilical blood flow (**BF**) was calculated using present functions on the ultrasound instrument. Abbreviations for the blood flow equation are as follows: mean velocity (**MnV**) and cross-sectional area of vessel (**CSA**). The blood flow equation is $BF \text{ (mL/min)} = MnV \text{ (cm/s)} \times CSA \text{ (cm}^2\text{)} \times 60 \text{ s}$.

2.3. Gestational day 130 blood sampling and necropsy procedures

Following intraoperative blood flow measurements, blood samples were taken simultaneously from the uterine vein (of the gravid horn; **UtV**) and the iliac artery (via a

catheter through the saphenous artery; maternal artery, **MA**). Thereafter, an incision was made in the gravid uterine horn and the umbilical cord was exposed. Blood samples were also collected simultaneously from the umbilical artery (**UmbA**) and the umbilical vein (**UmbV**). Blood (~ 60 mL) was collected into three 10-mL nonheparinized tubes (Becton Dickinson Vacutainer Systems). Serum was obtained by centrifugation (1,500 × g for 30 min) and stored at -20°C until further analysis.

After blood collection, the ewe was euthanized with an overdose of sodium pentobarbital (SleepAway, Fort Dodge Animal Health, Fort Dodge, IA). The fetus was then removed, weighed, and exsanguinated. Fetal curved crown-rump length (**CCR**) and abdominal girth were recorded. Fetal organs were harvested and weighed. The liver was snap frozen in liquid nitrogen and used for further analysis (see below). The placenta was collected, and placentomes were counted, dissected, and total placentome weight was recorded. The remaining interplacentomal membranes (i.e. fetal membranes) were weighed and recorded. The caruncular and cotyledonary tissues were separated and weighed. The maternal liver was removed, weighed, and a section removed and snap frozen for further analysis.

2.4. Amino acid analysis

Serum AA profiles were determined using an Ultra Performance Liquid Chromatograph (**UPLC**) as previously described by Lemley et al. (2013). Two-hundred fifty microliters of serum was deproteinized with 250 µL of 10% sulfosalicylic acid to which 250 µM norvaline was added as an internal standard. This mixture was vortexed and centrifuged for 5 min at 16,000 × g. Twenty microliters of supernatant was added to

60 μ L of borate buffer and sodium hydroxide solution as well as 20 μ L of MassTrac Amino Acid Analysis derivating reagent. The samples were then capped, mixed and heated in a digestion block at 55°C for 10 min. Samples were then injected into the UPLC. This method utilizes the MassTrac Amino Acid Analysis system for the full profile of AA in physiological fluids. Derivatization chemistry for physiological samples is a precolumn method and is based on a derivatizing reagent, 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate, which converts both primary and secondary AA to stable chromophores for UPLC detection.

2.5. Nitrite analysis

Total serum nitrites were determined using the QuantiChrom Nitric Oxide Assay Kit (BioAssay Systems, Hayward, CA). Briefly, following the manufacturer's recommendations, serum samples from the MA, UtV, UmbA, and UmbV were deprotenized and quantified following the reduction of total nitrates to nitrites using the Griess method and analyzed against a linear nitrites standard curve (0 to 100 μ M), with a sensitivity of 0.6 μ M and intra- and inter-assay coefficients of variation of 8.2% and 16.9%, respectively.

2.6. Sex steroid radioimmunoassay

The concentrations of estradiol-17 β and progesterone were determined following the procedures of Hart et al. (2014). Briefly, the progesterone and estradiol-17 β radioimmunoassay kits (Siemens Healthcare Diagnostics, Los Angeles, CA) and performed following the manufacturers' instructions. The steroid tracers were read in a

Packard gamma counter (Meriden, CT). The intra-assay coefficients of variation were 4.3% and 4.2% for estradiol-17 β and progesterone, respectively.

2.7. Hepatic steroid metabolizing enzyme activity

Liver homogenization and enzyme activity methods were according to Hart et al. (2014). Approximately, 100 mg of frozen liver tissue was weighed out, submerged in 2 mL of potassium phosphate (KPO₄) buffer (400 mM, pH = 7.4), and placed on ice for the remainder of the procedure. Samples were then homogenized using a glass Dounce homogenizer and then centrifuged at 10,000 $\times$ g for 10 min at 4°C. The supernatant (S9 fraction) was then placed into multiple aliquots to prevent freeze thaw cycles between the enzymatic activity assays. Supernatant fractions were stored at -80°C. Protein concentrations in the S9 fractions were determined using a Commassie Plus protein assay (Thermo Scientific, Rockford, IL).

Cytochrome P450 1A (CYP1A), 2C (CYP2C), and 3A (CYP3A) assay kits and NADPH regeneration system were purchased from Promega Corporation (Madison, WI). The activity of CYP1A, CYP2C and CYP3A were determined using the luminescent substrates luciferin CEE, luciferin H, and luciferin IPA, respectively. Following enzyme incubations plates were read using a Promega Multi-Plus plate reader using luminescence detection mode.

Aldo-keto reductase 1C (AKR1C) activity was determined using the methods of Lemley and Wilson (2010). Briefly, AKR1C was determined in S9 fractions using the specific substrate, 1-acenapthenol (Pfaltz & Bauer, Waterbury, CT). Enzymatic reactions contained 150 μ g of cytoplasmic protein, 250 μ M 1-acenapthenol, and 500 μ M

NADP. The 1-acenapthenol dependent reduction of NADP was standardized using the amount of cytoplasmic protein. The reduction of NADP was determined by measuring the amount of light absorbed at 340 nm for 10 min using a plate reader (Spectra Max Plus, Sunnyvale, CA). The extinction coefficient for NADPH (6,220 L/mol*cm) was used to calculate the rate of reduced NADP in pmol per min per mg of protein.

The uridine 5'-diphospho-glucuronosyltransferase (UGT) assay (Promega Corporation) was performed following the manufacturer's recommendations except for a reduction in substrate incubation time from 60 min to 10 min. This reduction resulted in an approximate 50% disappearance of the luminogenic substrate, which remained linear following enzyme dilutions. The UGT reaction mixture contained UGT multienzyme substrate consumed by UGT1A1, 1A8, 1A9, 1A10, 2B7, and 2B15 isozymes. The plate was read using a Promega Multi-Plus plate reader using luminescence detection mode.

2.8. Calculations

Average placentome weight was calculated by dividing total placentome weight by placentome number. Uterine and umbilical blood flow per fetal weight was calculated by dividing uterine and umbilical blood flow by fetal weight, respectively. Gravid uterine, fetal, and uteroplacental uptake of AA was calculated. The following abbreviations were used for AA uptake calculations: **[aa MA]** = AA concentration in the maternal artery (iliac artery via the saphenous catheter), **[aa UtV]** = AA concentration in the uterine vein (gravid horn), **[aa UmbV]** = AA concentration in umbilical vein, **[aa UmbA]** = AA concentration in umbilical artery, **Uterine BF** = uterine artery blood flow, and **Umbilical**

BF = umbilical blood flow. Equations for AA, serum nitrites and sex steroid flux are as follows: gravid uterine flux = ([aa MA nmol/mL] – [aa UtV nmol/nl]) × UtBF ml/min; fetal flux = ([aa UmbV nmol/mL] – [aa UmbA nmol/mL]) × UmbBF mL/min; uteroplacental flux = gravid uterine uptake – fetal uptake. The total serum nitrite, progesterone, and estradiol-17β difference between the uterine artery and uterine vein was calculated as [MA] - [UtV], and the total serum nitrite, progesterone, and estradiol-17β difference between the umbilical vein and umbilical artery was calculated as [UmbV] - [UmbA]. Enzymatic activity is expressed relative to liver homogenate protein concentrations, per g of liver, and relative to total liver weight. For CYP and UGT enzymes, the activity is expressed as relative light units (RLU) per min per mg of protein. In addition, CYP and UGT activity in the liver was determined by calculating activity per g of liver and multiplying by liver weight. For AKR1C activity, the rate of reduced NADP was expressed in pmol per min per mg of protein. In addition, total AKR1C activity in the liver was determined by calculating activity per g of liver and multiplying by liver weight.

2.9. Statistical analysis

Initial ewe body weight (i.e. day 100), final ewe body weight (i.e. day 130), placental measurements, blood flow measurements, AA and hormone concentrations, AA and hormone flux, fetal measurements, and enzymatic activity were analyzed using the ordinary least squares (GLM procedure of SAS [SAS Institute, Cary, NC]) with treatment in the model statement. Ewe body weight throughout the study was analyzed by repeated measures with the generalized least squares (MIXED procedure of SAS [SAS Institute]) with treatment, day of gestation, and the interaction of treatment × day

of gestation in the model. Means were separated using the PDIFF option of the LSMEANS statement. Least square means and SEM are reported.

3. Results

3.1. Ewe body weight

Day 100 (67.0 ± 2.5 kg) and final (68.8 ± 2.5 kg) ewe body weight did not differ ($P = 0.85$) among treatments. There was no treatment $\times$ day of gestation interaction ($P \geq 0.77$) for either ewe body weight or the percentage change in ewe body weight. There was an overall day effect ($P < 0.01$) on ewe body weight and the percentage change in ewe body weight, where ewes gained $2.46 \pm 0.78\%$ of their day 100 weight by day 130.

3.2. Uterine and umbilical blood flows

Uterine and umbilical blood flows are presented in Table 3. Maternal MP level tended ($P = 0.08$) to influence uterine blood flow, with MP60 ewes having greater uterine blood flow compared with MP100 ewes, with MP80 ewes being intermediate. When expressed as blood flow per fetal weight, MP60 ewes had greater ($P = 0.02$) uterine blood flow per fetal weight compared with MP80 and MP100 ewes, which were similar. There was no effect ($P \geq 0.14$) of maternal treatment on umbilical blood flow or umbilical blood flow per fetal weight. Moreover, there was no difference in maternal arterial blood pressure.

3.3. Placental and fetal measurements

Placental and fetal measurement data are presented in Table 3. The MP60 ewes had a lighter ($P = 0.03$) conceptus weight compared with MP100 ewes with MP80 ewes being intermediate (Table 3). Total placentome weight, placentome number, average placentome weight, total caruncular weight, total cotyledonary weight, and fetal membrane weight did not differ ($P \geq 0.12$) among the treatment groups. Fetuses from MP60 ewes were lighter ($P = 0.04$) compared with fetuses from MP100 ewes, with fetuses from MP80 ewes being intermediate. Fetal CCR was shorter ($P = 0.05$) and fetal girth was less ($P = 0.03$) in MP60 ewes compared with fetal CCR in MP100 ewes, with MP80 ewes being intermediate (Table 3).

Fetal organ data is presented in Table 4. Fetal brain, large intestine, perirenal fat, small intestine, and thyroid weights did not differ ($P \geq 0.11$) among treatment groups. Fetal heart, spleen, and stomach weights tended ($P \geq 0.06$ to $P \leq 0.09$) to be lighter in MP60 ewes compared with MP100 ewes. Fetal adrenal glands, kidneys, and liver were lighter ($P \leq 0.05$) in fetuses from MP60 ewes compared with fetuses from MP100 ewes, with MP80 ewes being intermediate. Fetal lung and pancreas weights were lighter ($P \leq 0.02$) in fetuses from MP60 ewes compared with fetuses from both MP80 and MP100 ewes, which were similar. When evaluated on an organ weight per fetal weight (g/kg) basis, only fetal brain weight per fetal weight tended ($P = 0.08$) to differ among treatments with MP60 ewes having a heavier brain weight per fetal weight compared with MP100 ewes, with MP80 ewes being intermediate. All other organ weights per fetal weight did not differ ($P \geq 0.14$). When evaluated on an organ weight per fetal brain weight (g/g) basis, only fetal lung weight per brain weight and fetal pancreas per brain weight differed where both were lighter ($P \leq 0.03$) in MP60 ewes compared with MP80

and MP100 ewes, which did not differ. Kidney weight per brain weight tended ($P = 0.06$) to be greater in MP100 than MP60 and MP80, which were similar.

3.4. Uterine, fetal, and uteroplacental flux of amino acids

All AA concentrations in the MA, UtV, UmbV, and UmbA can be found in Supplement Table 1. Amino acids that differed or tended to differ ($P \leq 0.08$) among treatment groups are depicted in Table 5. Glutamine concentrations in the MA, UmbV, and UmbA were not impacted ($P \geq 0.14$) by maternal protein level; however, glutamine concentrations in the UtV were greater ($P = 0.02$) in MP60 and MP80 ewes compared with MP100 ewes. Glycine concentrations were also greater ($P \leq 0.04$) in the MA, UtV, UmbV, and UmbA in the MP60 and MP80 ewes compared with MP100 ewes.

Isoleucine concentrations were similar across treatments for MA and UtV; however, tended to decrease ($P \leq 0.06$) in MP60 UmbV and UmbA compared with MP100.

Leucine concentrations in the MA and UmbV tended to decrease ($P = 0.06$) in MP60 compared with MP80 and MP100, with UtV being similar ($P = 0.12$) among all treatments. Leucine concentration in the UmbA was less ($P = 0.04$) in MP60 and MP80

ewes compared with MP100 ewes. Ornithine MA and UtV concentrations were greater ($P \leq 0.02$) in MP80 ewes compared with both MP60 and MP100 ewes; however, concentration in the UmbA and UmbV did not differ ($P \geq 0.66$) due to maternal

treatment. Serine concentrations in the UmbV and UmbA were similar ($P \geq 0.24$), while MA concentration tended to be reduced ($P = 0.08$) in MP100 compared to MP60 and MP80. Serine concentrations were greater ($P = 0.03$) in the UtV in MP60 and MP80 ewes compared with MP100 ewes. There was a tendency ($P = 0.08$) for tyrosine to be

decreased in MA from MP80 compared with MP100 ewes. Valine concentration in the MA and UtV were similar ($P \geq 0.39$) among treatment groups; however, in the UmbV, valine concentration was less ($P = 0.04$) in MP60 and MP80 ewes compared with MP100 ewes. In the UmbV, valine was less ($P = 0.03$) in MP60 ewes compared with MP100 ewes, with MP80 ewes being intermediate. The uterine, fetal, and uteroplacental uptakes of each AA did not differ among treatments (Supplement Table 1).

3.5. Total serum nitrites and sex steroids in maternal and fetal vascular beds

Total serum nitrites, the difference between nitrite concentration in the UtA and UtV, the difference between nitrite concentration in the UmbV and UmbA, and the uterine, fetal, and uteroplacental flux of total serum nitrites are presented in Table 6. Total serum nitrites in both the UtA and the UtV were less ($P \leq 0.05$) in the MP60 ewes compared with the MP100 ewes, with the MP80 ewes being intermediate. Uterine uptake of nitrites was greater ($P = 0.03$) in MP60 and MP80 ewes compared with MP100 ewes. In the umbilical vessels, there was a tendency ($P \leq 0.10$) for total serum nitrites to be less in MP60 and MP80 ewes compared with MP100 ewes; however, there was no difference ($P = 0.35$) among treatments in the fetal flux of serum nitrites. There was a tendency for greater ($P = 0.09$) uteroplacental uptake in the MP60 compared with MP80 and MP100 ewes.

Progesterone concentrations in MA and umbilical vessels were not impacted ($P > 0.10$) by maternal diet. There was a tendency ($P = 0.09$) however for progesterone in UtV to be decreased in MP60 vs. MP100 ewes. Estradiol-17 β was similar in most vessels, but there was a tendency ($P = 0.08$) for decreased concentrations in UmbA

from MP60 compared with MP80 ewes. The uteroplacental uptake was greater ($P = 0.02$) in MP100 compared with MP60 and MP80 ewes.

3.6. Hepatic steroid metabolizing enzyme activity

The effect of maternal MP levels on maternal hepatic steroid metabolizing enzyme activity are presented in Table 7. Maternal hepatic CYP1A activity was decreased ($P < 0.01$) in MP60 compared with MP80 and MP100 ewes. Maternal CYP2C activity was not different when expressed relative to protein concentrations or liver weight; however, CYP2C activity per g of liver tended to decrease ($P = 0.06$) in MP60 versus MP80 and MP100 ewes. Maternal CYP3A, AKR1C, and UGT activities were similar across all treatment groups.

The effect of maternal MP levels on fetal hepatic steroid metabolizing enzyme activity are presented in Table 8. Three out of eleven fetuses had detectable CYP1A activity, while no fetuses had detectable CYP2C activity. Fetal CYP3A and AKR1C activity was similar across all treatment groups. Fetal UGT activity per mg of protein and per g of liver was decreased ($P \leq 0.05$) in MP80 versus MP100 ewes. Fetal UGT activity relative to liver weight was decreased ($P = 0.02$) in MP60 and MP80 versus MP100 ewes.

4. Discussion

There are many well-established models of intrauterine growth restriction in sheep, but until now, no ruminant studies examined the effects of maternal MP during late gestation on uterine and umbilical blood flow, fetal organ weight, and AA

concentrations in uterine and umbilical vessels. This study indicates maternal MP restriction from day 100 to 130 of gestation does not negatively impact ewe weight or circulating AA concentrations; however, MP restriction during late gestation resulted in lighter fetuses despite the increased uterine blood flow per fetal weight and increase in certain AA.

In the present study, maternal dietary treatment did not alter ewe body weight from day 100 to day 130 of gestation. Van Emon et al. (2014) fed similar MP supplements to a larger group of ewes (n = 295) from day 100 until lambing and found ewes receiving less than the NRC recommendations for MP (60% MP) weighed less than ewes meeting MP recommendations (100% MP) on days 114 and 142 of gestation. Further, Van Emon et al. (2014) fed the dietary treatments from day 100 of gestation until lambing in a traditional production setting, whereas ewes were euthanized on day 130 of gestation in the present study. It is possible that if diets in the present study had been fed until lambing, differences in ewe body weight would have been observed.

In the present study, ewes receiving 60% of MP requirements had increased uterine blood flow per fetal weight (mL/min/fetal body weight) but similar placental weights compared with the ewes meeting MP requirements. Despite the greater uterine blood flow per fetal weight, fetuses were lighter and smaller in the MP60 ewes compared with the MP100 ewes. Diets in the current study were formulated to be isocaloric; however, fetal weight, CCR, and girth were all negatively altered by day 130 of gestation indicating maternal MP may play a key role in the growth of the developing fetus. The increase in uterine blood flow per fetal weight without an increase in placental

weight could indicate a compensatory mechanism in the MP60 ewes. This may support data from Van Emon et al. (2014) stating the increased uterine blood flow resulted in a catch-up growth, resulting in similar lamb birth weights. Van Emon et al. (2014) reported MP supplementation did not influence lamb birth weights, but did impact ewe lamb fertility (Van Emon et al., 2015). Perhaps the placenta is compensating for reduced maternal protein levels available by enhancing blood flow, and thereby enhancing the fetal growth trajectory, which could negatively impact physiology later in life.

Currently, no data exist on the effects of maternal MP during late gestation on uterine and umbilical blood flow in sheep. Lemley et al. (2012) recorded intraoperative uterine and umbilical blood flow measurements following a similar procedure to the current study using Doppler ultrasonography. In the Lemley et al. (2012) study, ewes received a control diet (100% of NRC requirements) or a globally-restricted diet (60% of NRC recommendations) from day 50 to day 130 of gestation. At day 130 of gestation, intraoperative ultrasound measurements showed a decrease in uterine blood flow in the restricted group; however, when expressed as uterine blood flow per fetal weight, there were no differences between the two treatments (Lemley et al., 2012). Umbilical blood flow and umbilical blood flow per fetal weight were not different at day 130 of gestation (Lemley et al., 2012), which is similar to the current study. Also similar to the current study, Lemley et al. (2012) observed lighter fetal weights in the restricted ewes compared with the control ewes, but similar placental weight between the two groups. It is important to remember Lemley et al. (2012) were studying the effects of a control diet versus a globally restricted diet, whereas in the current study, diets were formulated to be isocaloric and only differ in the level of MP. Taking this into account, it appears while

an overall nutrient restriction may lead to decreases in uterine blood flow (Lemley et al., 2012), specifically restricting MP may result in greater uterine blood flow per fetal weight. The current study suggests the decrease in fetal weight observed by Lemley et al. (2012) may be specifically due to a decrease in maternal protein in the diet.

While there is a wealth of literature examining the effects of maternal undernutrition on fetal and/or offspring organ weight, few studies have evaluated the effects of maternal MP in ruminants on fetal and/or offspring organ weight. In the current study, maternal protein restriction (MP60) tended to decrease fetal heart, spleen, and stomach weights compared with the control group (MP100), decreased the fetal kidney and liver weight compared with the controls, and decreased fetal lung and pancreas weight compared with both a moderately restricted MP group (MP80) and the control group. This indicates maternal MP restriction does result in intrauterine growth restriction. When expressed as fetal organ weight per fetal weight, only the fetal brain tended to be heavier in MP60 compared with MP100 ewes. This indicates a synchronous growth restriction in the MP60 ewes with the brain weight being spared.

Comparing our protein restriction data during late gestation to a global restriction during mid to late pregnancy (Lemley et al., 2013), we observed fewer differences; however, patterns for serine and glycine favored those provided adequate protein/energy compared with restricted ewes. Moreover, umbilical venous AA concentrations followed similar patterns if a ewe were protein restricted for 30 days (this study) or globally restricted for 80 days (Lemley et al., 2013) with the exception that glycine was elevated in MP60 ewes compared with MP100. Glycine in the umbilical vein was similar in globally restricted ewes compared with adequately fed ewes (Lemley et

al., 2013). Perhaps the observation that fewer AA were impacted within this study compared with the Lemley et al. (2013) paper is due to the duration of the restriction, the restriction of other nutrients, or both. Lekatz et al. (2011) reported in maternal venous and circulating fetal blood (collected from the fetal heart) that control ewes receiving 100% of global requirements did not differ in their concentrations of glutamate, glycine, leucine, ornithine, serine, and valine (AA different among treatments in the current study) from globally-restricted ewes at 130 days of gestation. This indicates while a global nutrient restriction during late gestation does not alter AA concentrations in maternal or fetal circulation (Lekatz et al., 2011), specifically restricting MP does alter AA concentrations.

Others have reported when ewes were globally restricted to 50% of NRC requirements from day 28 to 115 of gestation, concentrations of most AA in the uterine artery and umbilical vein were less compared with ewes fed to meet NRC requirements (Satterfield et al., 2010). Also, when ewes were fed 50% of NRC requirements from either day 28 to 78 of gestation or from day 28 to 135 of gestation, AA concentrations in the uterine artery and umbilical vein were decreased at days 78 and 135 of gestation (Kwon et al., 2004). It is difficult to compare the current study with Satterfield et al. (2010) and Kwon et al. (2004) for a few reasons. Satterfield et al. (2010) and Kwon et al. (2004) examined a 50% global nutrient restriction, whereas the current study is investigating isocaloric diets differing in MP. Further, Satterfield et al. (2010) fed the dietary treatments much earlier in gestation and collected blood samples at slaughter on day 115 of gestation. Kwon et al. (2004) also began dietary restriction early in gestation (day 28) and collected samples at days 78 and 135 of gestation. In the present study,

460 dietary treatments were fed during late gestation (day 90 to 130 of gestation), and blood
461 samples obtained under anesthesia at day 130 of gestation.

462 In the current study, the uterine, uteroplacental, and fetal flux of each AA were
463 calculated using AA concentrations in the uterine artery and vein, umbilical artery and
464 vein, and the uterine and umbilical blood flows. The calculated AA flux did not differ with
465 treatment; however, the uterine blood flow per fetal weight was greater in the MP60
466 ewes. Moreover, the current study did not investigate AA transporters, so it is still
467 possible that despite a lack of difference in AA flux, maternal MP restriction could still
468 alter nutrient availability to the fetus.

469 Uterine blood flow tended to be greater and uterine blood flow per fetal weight
470 was greater in MP60 ewes compared with control ewes, but total serum nitrites in the
471 uterine artery were less in MP60 ewes compared with control ewes. It was surprising
472 total serum nitrites were altered by maternal MP level but arginine levels were not. This
473 could indicate that while maternal diet does not alter the precursor arginine, key
474 enzymes such as nitric oxide synthase may be affected by MP level during late
475 gestation. Perhaps the increase in uterine blood flow is mediated through a mechanism
476 other than nitric oxide-dependent vasodilatory pathways. It would be useful to
477 investigate the vascular function of the maternal and fetal placental arteries to
478 determine what role nitric oxide is playing in the vasodilatory responses.

479 Previous work in sheep and cattle has shown increased concentrations of
480 progesterone and estradiol following reductions in dry matter intake (Parr et al., 1993;
481 Sangsritavong et al., 2002). Recently, our laboratory (Lemley et al., 2014) has reported
482 increased concentrations of progesterone from day 109 to 144 of gestation when

feeding 60% of nutrient requirements versus 100% from day 40 of pregnancy to term. Similarly, concentrations of estradiol were increased in nutrient restricted ewes on day 109 and 137 of gestation compared with control fed ewes. This alteration in peripheral steroid concentrations during pregnancy could be related to hepatic steroid metabolizing enzyme activity. For example, Hart et al. (2014) observed a negative correlation between CYP3A activity and peripheral concentrations of estradiol in nutrient restricted beef cattle, while AKR1C activity was negatively correlated with peripheral concentrations of progesterone. Similar studies have not been reported in sheep. In the current study, the decrease in uterine vein concentrations of progesterone in the MP60 ewes was not associated with an increase in hepatic steroid metabolizing enzyme activity. Moreover, we did not observe any concomitant changes in estradiol metabolizing enzymes, CYP1A and CYP3A with peripheral concentrations of estradiol. Therefore the alterations in uteroplacental flux of estradiol might be related to synthesis or local placental metabolism versus hepatic metabolism.

6. Conclusions

In conclusion, maternal MP restriction from day 90 to 130 of gestation does not appear to negatively affect ewe body weight, umbilical blood flow or AA flux. There was a modest increase in uterine blood flow in protein restricted ewes, potentially driven by nitric oxide, and alterations in branch-chained AA in circulation. Fetal weight was decreased in the most severely protein-restricted ewes, which indicates maternal MP significantly impacts fetal growth, at least up to day 130 of gestation. Interestingly, this decrease in fetal weight occurred despite an increase in uterine blood flow (expressed relative to fetal weight) and without changes in placental weight. Future studies are

needed to investigate possible compensatory mechanisms, such as increased blood flow and altered AA pathways, particularly the arginine-citrulline-nitric oxide pathway, which may occur in dams experiencing reduced MP during late gestation.

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Table 1. Nutrient composition of fescue straw used in the experiment¹.

Item	
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Diet, % DM	
DM, %	96.24
NEm, Mcal/kg	2.22
CP, % of DM	2.76
MP, % of DM	1.95
NDF, % of DM	80.17
ADF, % of DM	48.66
Ash, % of DM	6.00
<hr/>	

¹Ewes were fed fescue straw to limit MP intake

Table 2. Ingredient and nutrient composition of dietary supplements fed to ewes from day 100 to day 130 of gestation that provided differing levels of metabolizable protein (MP), but were similar in energy.

Item	Treatment ¹		
	MP60	MP80	MP100
Ingredient, % DM			
Corn	18.50	15.00	5.00
DDGS ²	7.00	20.00	30.00
Soyhulls	9.50	—	—
Nutrient composition			
DM, %	95.51	95.89	95.90
NEm, Mcal/kg	2.00	2.22	2.14
CP, % of DM	13.45	20.53	25.03
MP, % of DM	8.41	13.01	16.31
NDF, % of DM	33.61	32.11	40.79
ADF, % of DM	15.71	8.33	11.61
Ash, % of DM	3.17	3.50	4.38

¹Maternal diets (DM basis) were balanced for mature ewes during the last 4 weeks of gestation according to NRC (2007). Treatments included 60% of MP requirements (MP60), 80% of MP requirements (MP80), and 100% of MP requirements (MP100).

²Dried distillers grains plus solubles

Table 3. The effect of maternal metabolizable protein (MP) restriction from day 100 to 130 of gestation on uterine and umbilical blood flow measurements and conceptus measurements.

Measurement	Treatments ¹			SE	P-value
	MP60	MP80	MP100		
Maternal blood pressure, mmHg	150.5	124.8	113.6	13.5	0.19
Uterine BF ² (mL/min)	818^a	661^{ab}	447^b	105	0.08
Uterine BF (mL/min) / fetal weight (g)	0.42^a	0.22^b	0.12^b	0.07	0.02
Umbilical BF (mL/min)	387	425	328	90.9	0.70
Umbilical BF (mL/min) / fetal weight (g)	0.20	0.14	0.08	0.04	0.14
Conceptus weight ³ (g)	2643^a	3616^{ab}	4330^b	398	0.03
Total placentome weight (g)	491	560	482	47.9	0.41
Total placentome number	63.0	75.2	54.7	15.4	0.61
Average placentome weight (g)	8.45	7.55	12.15	2.38	0.36
Total CAR ⁴ weight (g)	103	118	70.9	27.0	0.45
Total COT ⁵ weight (g)	331	386	352	43.3	0.60
Fetal membrane weight (g)	202	218	402	69.6	0.12
Fetal weight (g)	2151^a	3056^{ab}	3848^b	423	0.04
Fetal CCR ⁶ (cm)	50.8^a	55.1^{ab}	62.5^b	3.02	0.05
Fetal girth (g)	28.2^a	31.8^{ab}	35.6^b	1.61	0.03

¹Maternal diets (DM basis) were balanced for mature ewes bearing twins during the last 4 weeks of gestation according to NRC (2007). Treatments included 60% of MP requirements (MP60), 80% of MP requirements (MP80), and 100% of MP requirements (MP100). Diets were fed from day 100 to 130 of gestation.

²Blood flow

³Placental weight + fetal weight

⁴Caruncular tissue

⁵Cotyledonary tissue

⁶Curved crown-rump length

^{ab}LSMeans $\pm$ SEM within a row differ, $P \leq 0.05$

Table 4. The effect of maternal metabolizable protein (MP) restriction from day 100 to 130 of gestation on fetal organ weights and fetal organ weight expressed relative to fetal weight.

Measurement	Treatments ¹			SEM	P-value
	MP60	MP80	MP100		
Adrenal weight (g)	0.22^a	0.30^{ab}	0.34^b	0.03	0.05
g / kg ² fetal weight	0.10	0.10	0.09	0.01	0.51
g / g ³ brain weight	0.006	0.007	0.007	0.001	0.30
Brain weight (g)	36.8	44.2	45.6	4.47	0.30
g / kg fetal weight	17.9^a	14.9^{ab}	11.9^b	1.69	0.08
g / g brain weight	1.00	1.00	1.00	0.001	0.99
Heart weight (g)	15.4^a	21.1^{ab}	26.0^b	2.98	0.07
g / kg fetal weight	7.12	7.02	6.76	0.30	0.66
g / g brain weight	0.42	0.47	0.57	0.06	0.24
Kidney weight (g)	16.7^a	20.3^{ab}	25.6^b	1.97	0.03
g / kg fetal weight	8.00	6.87	6.63	0.52	0.15
g / g brain weight	0.47^a	0.45^a	0.57^b	0.03	0.06
Large intestine weight (g)	6.03	8.24	9.22	1.05	0.11
g / kg fetal weight	2.79	2.78	2.40	0.18	0.25
g / g brain weight	0.17	0.18	0.21	0.02	0.39
Liver weight (g)	71.0^a	78.7^{ab}	102.0^b	4.71	0.01
g / kg fetal weight	34.5	28.1	26.5	4.42	0.37
g / g brain weight	1.96	1.84	2.26	0.22	0.39
Lung weight (g)	79.8^a	116^b	139^b	13.0	0.02
g / kg fetal weight	38.5	38.5	36.2	2.47	0.73
g / g brain weight	2.17^a	2.60^b	3.10^b	0.20	0.03
Pancreas weight (g)	2.08^a	3.24^b	3.88^b	0.30	0.01
g / kg fetal weight	1.03	1.09	1.01	0.11	0.86
g / g brain weight	0.06^a	0.07^b	0.08^b	0.001	0.01
Perirenal fat weight (g)	14.4	18.5	16.9	2.71	0.49
g / kg fetal weight	6.55	6.47	4.43	0.79	0.14
g / g brain weight	0.75	0.42	0.33	0.05	0.51
Small intestine weight (g)	20.3	32.5	30.4	8.66	0.52
g / kg fetal weight	9.10	10.63	8.07	1.99	0.63
g / g brain weight	0.53	0.72	0.64	0.15	0.62
Spleen weight (g)	4.32^a	5.77^b	5.34^b	0.47	0.09
g / kg fetal weight	2.10	2.17	1.39	0.47	0.44
g / g brain weight	0.12	0.14	0.12	0.02	0.69
Stomach weight (g)	15.7^a	21.7^{ab}	27.3^b	3.01	0.06
g / kg fetal weight	7.29	7.25	7.08	0.45	0.94
g / g brain weight	0.42	0.49	0.60	0.55	0.10
Thyroid weight (g)	0.51	0.68	0.83	0.13	0.24
g / kg fetal weight	0.24	0.22	0.22	0.03	0.72
g / g brain weight	0.01	0.01	0.02	0.001	0.26

¹Maternal diets (DM basis) were balanced for mature ewes during the last 4 weeks of gestation according to NRC (2007). Treatments included 60% of MP requirements (MP60), 80% of MP requirements (MP80), and 100% of MP requirements (MP100). Diets were fed from day 100 to 130 of gestation.

²Each organ weight is also expressed as ratio of organ weight (g) per fetal weight (kg)

³Each organ weight is also expressed as ratio of organ weight (g) per fetal brain weight (g)

^{ab}LSMeans $\pm$ SEM within a row differ, $P \leq 0.05$

Table 5. The effect of maternal metabolizable protein (MP) restriction on select amino acid concentrations in the maternal artery, uterine vein, umbilical vein, and umbilical artery on day 130 of gestation.

Amino acid (nmol/mL)	Treatments ¹			SEM	<i>P</i> -value
	MP60	MP80	MP100		
Glutamine					
MA ²	265	255	226	13.2	0.14
UtV ³	246^a	229^a	129^b	11.4	0.02
UmbV ⁴	704	739	647	76.6	0.68
UmbA ⁵	615	589	548	57.1	0.68
Glycine					
MA	408^a	369^a	226^b	46.1	0.04
UtV	417^a	367^a	178^b	60.8	0.04
UmbV	648^a	765^a	429^b	47.9	0.04
UmbA	595^a	642^a	388^b	56.7	0.02
Isoleucine					
MA	41.3	59.8	56.8	7.70	0.18
UtV	37.1	54.0	45.9	6.79	0.19
UmbV	59.9^a	81.1^a	126^b	17.7	0.06
UmbA	50.0^a	69.0^{ab}	113^b	16.0	0.05
Leucine					
MA	58.3^a	89.0^b	97.9^b	11.3	0.06
UtV	52.5	81.3	78.6	10.7	0.12
UmbV	126^a	160^a	244^b	31.0	0.06
UmbA	101^a	132^a	219^b	27.9	0.04
Ornithine					
MA	19.3^a	35.0^b	20.0^a	2.60	0.01
UtV	16.9^a	28.8^b	13.3^a	2.54	0.02
UmbV	52.4	50.4	56.6	5.17	0.66
UmbA	59.7	49.5	54.6	4.98	0.70
Serine					
MA	45.2^a	42.9^a	33.0^b	3.58	0.08
UtV	40.9^a	38.0^a	27.3^b	2.97	0.03
UmbV	640	742	503	98.0	0.24
UmbA	663	734	520	94.8	0.29
Tyrosine					
MA	26.8^{ab}	19.6^a	30.6^b	3.24	0.08
UtV	24.7	16.6	25.5	3.46	0.14
UmbV	106	66.4	132	22.1	0.13
UmbA	94.9	57.5	119	20.2	0.12
Valine					
MA	69.9	90.5	87.9	12.4	0.39
UtV	63.2	82.8	71.2	11.2	0.40
UmbV	175^a	235^a	323^b	36.4	0.04
UmbA	153^a	214^{ab}	302^b	33.3	0.03

¹Maternal diets (DM basis) were balanced for mature ewes bearing twins during the last 4 weeks of gestation according to NRC (2007). Treatments included 60% of MP requirements (MP60), 80% of MP requirements (MP80), and 100% of MP requirements (MP100).

²MA = maternal artery (saphenous artery)

³UtV = uterine vein

⁴UmbV = umbilical vein

^bUmbA = umbilical artery

^{ab}LSMeans $\pm$ SEM within a row differ, $P \leq 0.05$

Table 6. The effect of maternal metabolizable protein (MP) level on total serum nitrite, progesterone, and estradiol-17 β concentration¹ in the uterine artery, uterine vein, umbilical artery, and umbilical vein of ewes on day 130 of gestation.

	Treatments ¹				
Vessel	MP60	MP80	MP100	SEM	P-value
Nitrate, μ M					
Uterine artery ²	5.65 ^a	10.7 ^{ab}	14.5 ^b	2.33	0.05
Uterine vein	4.65 ^a	10.3 ^{ab}	16.1 ^b	2.28	0.02
[Uterine artery] - [Uterine vein] ³	1.00 ^a	0.30 ^a	-1.57 ^b	0.60	0.03
Uterine flux ⁴	863 ^a	204 ^a	-704 ^b	348	0.03
Umbilical artery	44.7 ^a	47.9 ^a	90.9 ^b	14.3	0.08
Umbilical vein	49.1	54.2	97.7	15.9	0.10
[Umbilical vein] - [Umbilical artery] ⁵	4.39	6.35	6.86	2.04	0.62
Fetal flux ⁶	1619	2960	3409	1078	0.35
Uteroplacental flux ⁷	-756 ^a	-2757 ^{ab}	-3740 ^b	1033	0.09
Progesterone, ng/ml					
Uterine artery ²	75.24	101.44	123.25	20.43	0.25
Uterine vein	165.16 ^a	319.55 ^b	391.79 ^b	69.65	0.09
[Uterine artery] - [Uterine vein] ³	-90.00	-218.00	-268.33	81.63	0.27
Uterine flux ⁴	-67226	-126853	-113394	33829	0.37
Umbilical artery	19.02	10.89	15.87	4.03	0.31
Umbilical vein	15.90	7.69	11.14	4.26	0.34
[Umbilical vein] - [Umbilical artery] ⁵	3.11	3.20	4.73	1.16	0.54
Fetal flux ⁶	1163	1439	1275	596	0.90
Uteroplacental flux ⁷	-68389	-128292	-90552	41021	0.39
Estradiol-17 β , pg/ml					
Uterine artery ²	29.83	47.30	60.48	13.71	0.29
Uterine vein	32.48	51.07	44.11	7.06	0.16
[Uterine artery] - [Uterine vein] ³	-2.75	-4.00	16.33	7.64	0.15
Uterine flux ⁴	-2249	-2415	5760	2816	0.11
Umbilical artery	24.34 ^a	36.51 ^b	29.44 ^{ab}	3.71	0.08
Umbilical vein	28.47	34.68	31.43	3.59	0.41
[Umbilical vein] - [Umbilical artery] ⁵	-4.12	1.83	-1.99	3.11	0.34
Fetal flux ⁶	-1823	1247	-860	1712	0.26
Uteroplacental flux ⁷	-426 ^a	-3662 ^a	9385 ^b	2920	0.02

¹Maternal diets (DM basis) were balanced for mature ewes during the last 4 weeks of gestation according to NRC (2007). Treatments included 60% of MP requirements (MP60), 80% of MP requirements (MP80), and 100% of MP requirements (MP100).

²Uterine artery samples taken via a catheter through the saphenous artery to the internal iliac artery.

³[Uterine artery] - [Uterine vein] = the difference in concentration between the uterine artery and the uterine vein.

⁴Gravid uterine flux (respective units/ml/min) = ([total uterine artery] - [total uterine vein]) $\times$ uterine blood flow, ml/min.

⁵[Umbilical vein] - [Umbilical artery] = the difference in concentration between the umbilical vein and umbilical artery.

⁶Fetal flux (respective units/ml/min) = ([total umbilical artery] - [total umbilical artery]) $\times$ umbilical blood flow, ml/min.

⁷Uteroplacental flux (respective units/ml/min) = gravid uterine uptake - fetal uptake.

^{ab}LSMeans $\pm$ SEM within a row differ, $P \leq 0.05$

Table 7. The effect of maternal metabolizable protein (MP) level on liver mass and hepatic steroid metabolizing enzyme activity¹ of ewes on day 130 of gestation.

Dependent variable	MP60 ²	MP80	MP100	SEM	P-values
Liver weight, g	595.7	611.6	651.6	46.0	0.66
CYP1A					
RLU/(min*mg protein)×10 ³	28.97^a	52.83^b	52.30^b	3.42	<0.01
RLU/(min*g liver)×10 ³	7.78^a	15.10^b	14.21^b	7.60	<0.01
RLU/(min*liver) ×10 ⁴	459.7^a	925.9^b	929.1^b	82.6	<0.01
CYP2C					
RLU/(min*mg protein)	3,195	4,810	5,272	693	0.11
RLU/(min*g liver)	847.8^a	1,371.2^b	1,444^b	181.5	0.06
RLU/(min*liver) ×10 ⁴	50.3	83.9	97.4	15.4	0.11
CYP3A					
RLU/(min*mg protein)×10 ⁴	113.41	156.76	185.00	30.13	0.24
RLU/(min*g liver)×10 ⁶	80.77	119.30	136.31	23.08	0.22
RLU/(min*liver) ×10 ⁹	48.1	73.3	92.7	18.6	0.25
AKR1C					
pmol/(min*mg protein)	58.22	69.39	40.65	11.72	0.24
pmol/(min*g liver)	4,273	5,272	2,983	932.2	0.24
pmol/(min*liver) ×10 ⁵	25.5	32.2	19.8	6.0	0.33
UGT					
RLU/(min*mg protein)×10 ⁵	14.51	11.04	13.04	3.01	0.65
RLU/(min*g liver)×10 ⁶	104.14	84.48	93.43	21.03	0.75
RLU/(min*liver) ×10 ⁹	62.2	52.0	62.6	14.7	0.81

¹Cytochrome P450 1A (CYP1A), cytochrome P450 2C (CYP2C), cytochrome P450 3A (CYP3A), aldo-keto reductase 1C (AKR1C), uridine 5'-diphospho-glucuronosyltransferase (UGT), relative light units (RLU).

²Maternal diets (DM basis) were balanced for mature ewes during the last 4 weeks of gestation according to NRC (2007). Treatments included 60% of MP requirements (MP60), 80% of MP requirements (MP80), and 100% of MP requirements (MP100).

^{ab}LSMeans ± SEM within a row differ, $P \leq 0.05$

Table 8. The effect of maternal metabolizable protein (MP) level on fetal hepatic steroid metabolizing enzyme activity¹ on day 130 of gestation.

Dependent variable	MP60	MP 80	MP100	SEM	<i>P</i> -values
CYP1A					
RLU/(min*mg protein)	ND	199.18	36.67	141.74	
RLU/(min*g liver)	ND	55.69	10.33	39.63	
RLU/(min*liver)	ND	4636.64	1133.47	3316.75	
CYP2C					
RLU/(min*mg protein)	ND	ND	ND	ND	
RLU/(min*g liver)	ND	ND	ND	ND	
RLU/(min*liver)	ND	ND	ND	ND	
CYP3A					
RLU/(min*mg protein)×10 ³	11.64	3.67	11.65	8.74	0.71
RLU/(min*g liver)×10 ³	750.30	266.96	785.94	567.35	0.73
RLU/(min*liver)×10 ⁶	53.9	21.1	77.3	478.0	0.68
AKR1C					
pmol/(min*mg protein)	15.57	3.12	1.88	5.97	0.20
pmol/(min*g liver)	1,027.26	231.01	135.14	379.52	0.19
pmol/(min*liver)	18,080	74,536	13,120	27,306	0.20
UGT					
RLU/(min*mg protein)×10 ⁴	8.35^{ab}	3.78^a	16.60^b	3.16	0.04
RLU/(min*g liver)×10 ⁶	5.80^{ab}	2.81^a	11.95^b	124.7	0.05
RLU/(min*liver)×10 ⁶	415.0^a	228.4^a	1,191.1^b	204.4	0.02

¹Cytochrome P450 1A (CYP1A), cytochrome P450 2C (CYP2C), cytochrome P450 3A (CYP3A), aldo-keto reductase 1C (AKR1C), uridine 5'-diphospho-glucuronosyltransferase (UGT), relative light units (RLU).

²Maternal diets (DM basis) were balanced for mature ewes during the last 4 weeks of gestation according to NRC (2007). Treatments included 60% of MP requirements (MP60), 80% of MP requirements (MP80), and 100% of MP requirements (MP100).

^{ab}LSMeans ± SEM within a row differ, *P* ≤ 0.05